IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re the Application of: SOPHIE GAUBERT et al	Group Art Unit: 1615 Examiner: G. Kishore
Serial No.: 10/069,050	
Filed: March 7, 2002	

For: COMPOSITION TO BE ADMINISTERED THROUGH MUCOUS MEMBRANE

DECLARATION UNDER 37 CFR §1.132

Honorable Commissioner for Patents
PO Box 1450
Alexandria, VA 22313-1450
Sir:

I, Rene Laversanne, do hereby declare as follows:

I am a named inventor of the above-identified patent application.

The experiments described thereafter have been set-up in order to compare as delivery systems of antigens, for administration through mucous membrane, multilamellar vesicles as described in US application N°10/069,050 and classical liposomes as used in de Haan et al (vaccines 13, n° 2, pp 155-162, 1995).

It is the position of the Applicants that the claimed system does not need any specific lipid, and in particular does not need negatively charged lipids, while the classical liposomes are known to amplify the immune response elicited by antigen only in presence of negatively charged lipids.

The experiments were performed on mice using the protein FHA from *Bordetella pertussis*, with a nasal administration, and measuring

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the titer in IgG found in serum and in IgA found in broncho-alveolar lavages. For each system (classical liposomes and multilamellar vesicles), two formulations have been used: one made only from phosphatidyl choline (PC) which is neutral at the pH of the experiment, and the other made of a mixture of phosphatidyl choline and dicetyl phosphate (DP) which is negatively charged. Zeta potential and mean size measurements have been performed in order to check the actual electrical charge of each formulation.

Further experiments have been carried out to study the effect of the volume of the nasal instillation. Indeed, it is the position of the Applicants that the amplification of the immune response observed with liposomes is at least partially due to the fact that with the volume of instillation (50 µL) which is used according to the cited publication, the antigen reaches the lungs and therefore it is no longer a real nasal immunization.

Experimental

Sample preparation

FHA the purified filamentous protein from *Bordetella pertussis* was kindly provided by Sanofi-Aventis Pasteur (Marcy l'Etoile, France). It was used as a 1 mg/mL solution in PBS. Phosphatidyl Choline (lecithin, PC) was obtained from Phospholipid GmbH (Köln, Germany) as Phospholipon® 90G. Macrogol Oleate was purchased from SEPPIC (Castres, France) as Simulsol® 2599. The other components were provided by Sigma-Aldrich.

Each sample has been prepared in order to administrate a total amount of FHA antigen to each mouse of 3 µg.

The classical liposomes have been prepared using a method similar to the one described in de Haan et al. Lipid formulation consisted of either 50% of lecithin and 50% of cholesterol, or 40% lecithin, 10% of dicetyl phosphate and 50% cholesterol (cf. table 1 below). Separation of non encapsulated antigen from encapsulated antigen has been done using gel permeation chromatography on a Sephadex G-25 column using PBS as eluent. A protein assay (Lowry) has shown that the encapsulated antigen was about 80% of the initial amount of antigen incorporated. Starting from this liposomes stock suspension, dilution has been made in PBS to obtain the right concentration of antigen for nasal immunization.

Multilamellar vesicles have been prepared following a simplified version of the experimental method described in example 1 of the US application N°10/069,050. Formulation consisted of 65% of a mixture of lipids and surfactant according to table1, and 35% of aqueous phase containing the antigen. All the constituents were mixed and left overnight at 37°C with stirring intervals followed by shearing and dispersion. The final dispersion in PBS has been adjusted according to the antigen concentration needed for the nasal instillation.

Solutions of FHA diluted in PBS have been prepared at the same concentration as the encapsulated samples to be used as control.

Table 1: Lipid composition

	Uncharged Liposomes UL	***Negative Liposomes NL***	***Uncharged Vesicles UV***	***Negative Vesicles NV***
Phospholipon P90G	50 %	40 %	85 %	75 %
Cholesterol	50 %	50 %		
Simulsol 2599			15 %	15 %
Dicetyl Phosphate		10 %		10 %

Table 2: Samples identification

Designation	***Description***	***Concentration in FHA***
UL 1	Uncharged liposomes 20 µL/nostril	3 µg in 40 µL
UL 2	Uncharged liposomes 50 µL/nostril	3 µg in 100 µL
NL 1	Negative liposomes 20 µL/nostril	3 µg in 40 µL
NL 2	Negative liposomes50 µL/nostril	3 µg in 100 µL
UV 1	Uncharged vesicles 20 µL/nostril	3 µg in 40 µL
UV 2	Uncharged vesicles 50 µL/nostril	3 µg in 100 µL
NV 1	Negative vesicles 20 µL/nostril	3 µg in 40 µL
NV2	Negative vesicles 50 µL/nostril	3 µg in 100 µL
Solution 1	Solution, 20 µL/nostril	3 µg in 40 µL
Solution 2	Solution, 50 µL/nostril	3 µg in 100 µL

Sample characterization

Liposomes and multilamellar vesicles have been characterized using a Malvern ZetaSizer 3000HS which gives both the zeta potential and the size. It has been verified that the final dilution had no influence on the size. For measurements purpose, the samples have been diluted in pure water, in order to minimize the effect of ionic strength on the zeta potential. Results are given in the following table.

Table 3: physical characterization of the samples

Sample	***UL***	***NL***	***UV***	***NV***
Average size	0,15 µm	0,12 µm	0,3 µm	0,35 µm
ζ potential	-2 mV	- 40 mV	1 mV	-35 mV

Immunizations, sample collections and assay

6 to 8 weeks old female Balb/c mice (Charles River, France) have been used. Mice have been divided in 10 groups of 5 mice each group receiving a formulation as described in Table 2. Immunization have been done at day 0 and day 30, on slightly anesthetized mice. For samples with a titer of 3 µg in 40 µL, each mouse received 20 µL in each nostril, while for samples with a titer of 3 µg in 100 µL the instillation volume was 50 µL per nostril.

The animals were sacrificed at day 60 (one month after the last immunization) and serum and broncho alveolar lavages were collected as described in example 1 of US application N°10/069,050.

Antibody assay has been performed following the same procedure as described in the above mentioned patent application.

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Results

Results are given in Table 4 and in Figure 1 and 2.

Table 4: Titer in antibody (IgG and IgA) obtained after nasal immunization using the various formulations.

		IgG (serum)		IgA (BAL)	
		Average	SD	Average	SD
Uncharged liposomes 20 µL/nostril	UL1	1 831	1 157	70	39
Negative liposomes 20 µL/nostril	NL1	211 719	116 482	1 501	997
Uncharged vesicles 20 µL/nostril	UV1	1 562 500	956 832	11 475	4 395
Negative vesicles 20 µL/nostril	NV1	1 193 750	514 630	12 695	3 220
Solution, 20 µL/nostril	S1	1 183	676	4	3
Uncharged liposomes 50 µL/nostril	UL2	16 016	4 996	195	128
Negative liposomes 50 µL/nostril	NL2	1 328 125	1 090 956	19 824	7 825
Uncharged vesicles 50 µL/nostril	UV2	2 031 250	1 048 157	9 570	4 205
Negative vesicles 50 µL/nostril	NV2	2 656 250	2 181 913	22 656	15 459
Solution, 50 µL/nostril	S2	3 833	1 322	17	10

The same results are presented in the following figures.

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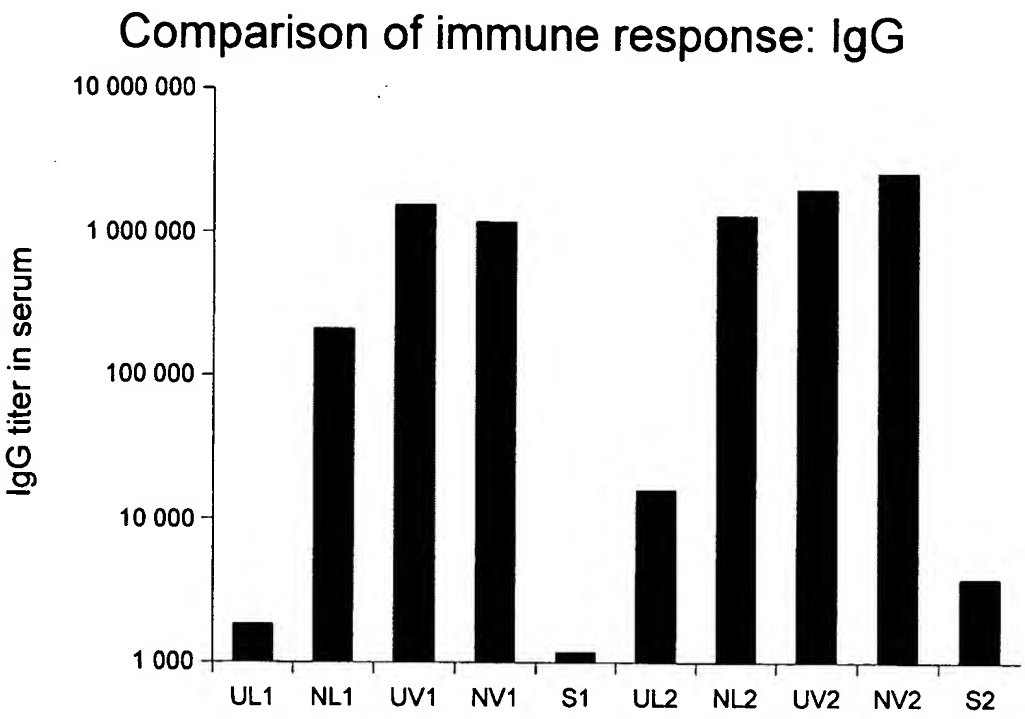

Figure 1: Comparison of the IgG antibody elicited against FHA from Bordetella pertussis obtain with different formulation of the antigen FHA (the designation of the groups is given in Table 2)

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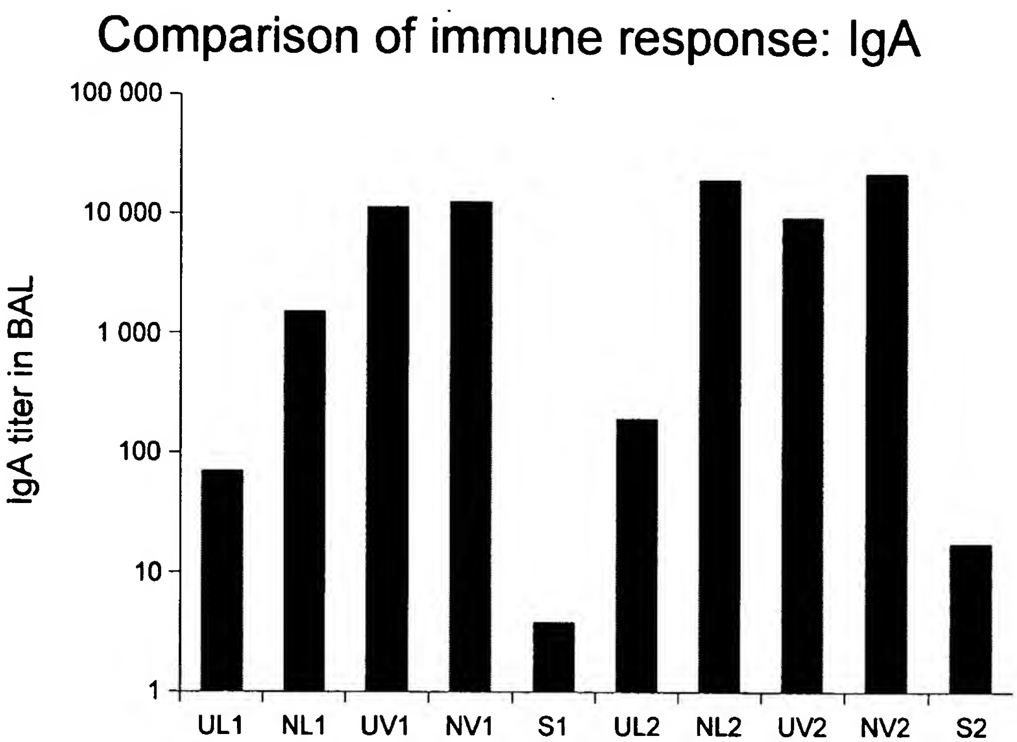

Figure 2: Comparison of the IgA antibody elicited against FHA from Bordetella pertussis obtain with different formulation of the antigen FHA (the designation of the groups is given in Table 2)

Discussion

The effect of the electrical charge of the lipids on the immune response is clearly illustrated on the liposomes, as expected from the literature, for both the systemic (IgG) and the local (IgA) response. The difference, for the same volume of instillation, between charged and uncharged liposomes reaches a factor higher than 100 for IgG and higher than 10 for IgA. On the contrary, the multilamellar vesicles described in US application N°10/069,050 show very little difference linked to the electrical charge of the lipids. The difference is small and lower than the dispersion of the results among each group.

On the other hand one can observe that the "volume effect" due to the volume of each instillation in the nostril is also more visible on the response elicited by the liposomes compared with the response obtained with the multilamellar vesicles of the invention. This means that the amplification of the immune response observed with liposomes and described in the literature is at least partially due to the antigen reaching

the lungs. For safety reason, this is to be avoided in an actual immunization scheme for Humans.

The stronger amplification obtained with the multilamellar vesicles compared with classical liposomes at small volume of instillation and the smaller influence of the volume of instillation could be related to a size effect of the particles (the liposomes are smaller). However, the effect observed on the electrical charge cannot be explained with such a consideration. This is a surprising effect of the specific structure of the multilamellar vesicles. The explanation may come from the better stability of the multilamellar vesicles (with less sensitivity to enzymatic destruction) but this would need much more experiments to demonstrate.

I further declare that all statements made by me herein are true and all statements made on information and belief are believed to be true, and that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and may jeopardize the validity of the application or any patent issued thereon.

Feb 19, 2007

Date

Rene Laversanne